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Targeted Gene Delivery to Mammalian Cells by Filamentous Bacteriophage

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We report that prokaryotic viruses can be re-engineered to infect eukaryotic cells resulting in expression of a reporter gene inserted into the bacteriophage genome. Phage capable of binding mammalian cells expressing the growth factor receptor ErbB2 and undergoing receptor-mediated endocytosis were isolated by selection of a phage antibody library on breast tumor cells and recovery of infectious phage from within the cell. As determined by immunofluorescence, F5 phage were efficiently endocytosed into 100% of ErbB2 expressing SKBR3 cells. To achieve reporter gene expression, F5 phage were engineered to package the green fluorescent protein (GFP) reporter gene driven by the CMV promoter. These phage when applied to cells underwent ErbB2-mediated endocytosis leading to GFP expression. GFP expression occurred only in cells overexpressing ErbB2, was dose-dependent reaching, 4% of cells after 60 hours and was detected with phage titers as low as 2.0×10^7 cfu/ml (500 phage/cell). The results demonstrate that bacterial viruses displaying the appropriate antibody can bind to mammalian receptors and utilize the endocytic pathway to infect eukaryotic cells, resulting in expression of a reporter gene inserted into the viral genome. This represents a novel method to discover targeting molecules capable of delivering a gene intracellularly into the correct trafficking pathway for gene expression by directly screening phage antibodies. This should significantly facilitate the identification of appropriate targets and targeting molecules for gene therapy or other applications where delivery into the cytosol is required. This approach can be adapted to directly select, rather than screen, phage antibodies for targeted gene expression. The results also demonstrate the potential of phage antibodies as an *in vitro* or *in vivo* targeted gene delivery vehicle.

Keywords: endocytosis; ErbB2; gene therapy; phage display; single chain Fv antibodies

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Introduction

Widespread application of gene therapy requires the ability to target a therapeutic gene to the appropriate cell or tissue type with high efficiency (Michael & Curiel, 1994). Targeting of retroviral vectors has been reported by inserting receptor ligands or single chain Fv (scFv) antibody fragments into the viral envelope protein (Kasahara *et al.*, 1994; Somia *et al.*, 1995). Targeting of adenoviral vectors has been achieved by use of "adapter" fusion molecules consisting of an antibody fragment that binds the adenoviral knob and a cell targeting molecule such as a receptor ligand or antibody (Douglas *et al.*, 1996; Watkins *et al.*, 1997). Targeting of non-viral vectors using cell surface receptor ligands or antibodies has also been reported (Fominaya & Wels, 1996; Michael & Curiel, 1994). All of these approaches depend on the use of targeting molecules that bind a cell-surface receptor, resulting in internalization of the gene delivery vehicle with subsequent delivery of the DNA to the nucleus. Identification of appropriate targeting molecules has largely been performed by individually screening receptor ligands or antibodies. In the case of scFv antibody fragments, this typically requires construction of the scFv from the

Abbreviations used: ECD, extracellular domain; CMV, cytomegalovirus; FGF, fibroblast growth factor; GFP, green fluorescent protein; MFI, mean fluorescent intensity; cfu, colony-forming units.

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V-genes of a hybridoma, construction of the targeted gene delivery vehicle, and *in vitro* evaluation of targeting ability.

More recently, it has proven possible to directly select peptides and antibody fragments binding cell-surface receptors from filamentous phage libraries (Andersen *et al.*, 1996; Barry *et al.*, 1996; Cai & Garen, 1995; de Kruif *et al.*, 1995; Marks *et al.*, 1993). This has led to a marked increase in the number of potential targeting molecules. The ability of bacteriophage to undergo receptor-mediated endocytosis (Hart *et al.*, 1994; Barry *et al.*, 1996; Becerril *et al.*, 1999) indicates that phage libraries can be selected not only for cell binding but also for internalization into mammalian cells. If the phage single-stranded phage genome can be transcribed and translated, then it should prove possible to screen or select for phage that bind receptors in a manner that leads to endocytosis and delivery of the phage genome into the correct trafficking pathway leading to expression. It has been shown that phage can enter mammalian cells after chemical alteration of the cell membrane leading to reporter gene expression (Okayama & Berg, 1985; Yokoyama-Kobayashi & Kato, 1993). More recently, Larocca *et al.* (1998) showed that indirect bacteriophage-mediated gene delivery could occur by targeting biotinylated phage *via* streptavidin and biotinylated fibroblast growth factor (FGF) to mammalian cells expressing FGF receptor.

Here, we show that filamentous phage displaying the anti-ErbB2 scFv F5 as a genetic fusion with the phage minor coat protein pIII can directly infect mammalian cells expressing ErbB2 leading to expression of a reporter gene contained in the phage genome. This offers a new way to discover targeting molecules for intracellular drug delivery or gene therapy by directly screening phage antibodies to identify those capable of undergoing endocytosis and delivering a gene intracellularly into the correct trafficking pathway for gene expression. This should significantly facilitate the identification of appropriate targets and targeting molecules for gene therapy or other applications where delivery into the cytosol is required. We also discuss how this approach might be used to directly select phage antibodies for targeted gene expression. Finally, we discuss the potential for use of phage antibodies themselves for *in vitro* or *in vivo* targeted gene delivery vectors.

Results

Internalization of ErbB2 binding monovalent and multivalent F5 phage particles by ErbB2 xpressing cells

We isolated the anti-ErbB2 scFv-F5 from a library of scFv displayed on the surface of bacteriophage as fusions to pIII (Sheets *et al.*, 1998) by selection on ErbB2 expressing SKBR3 breast tumor cells and recovery of infectious phage from within the cell (M.-A. *et al.*, unpublished results). This selection strategy (Hart *et al.*, 1994; Barry *et al.*, 1996; Becerril *et al.*, 1999) was employed to select scFv capable of undergoing endocytosis upon receptor binding. When the pHEN-F5 phagemid vector is rescued with VCS-M13 helper phage, the resulting virus particles (F5-phagemid) display an average of one copy of scFv-pIII fusion protein and three to four copies of the wild-type pIII minor coat protein from the helper phage (Marks *et al.*, 1992). As a result, the phagemid bind monovalently. To improve the binding of the virus particles to ErbB2 expressing cells, multivalent phage antibodies were created by subcloning the F5 scFv DNA into the phage vector fd-Sfi/Not for fusion with the pIII protein. Virus particles, referred to as fd-F5 phage, display four to five copies of scFv-pIII fusion protein (Marks *et al.*, 1992).

To determine whether F5 phage antibodies could be internalized by mammalian cells, SKBR3 cells overexpressing ErbB2 were incubated for 16 hours with fd-F5 phage (10^9 cfu/ml), F5 phagemid (10^{11} cfu/ml), or with phagemids displaying an irrelevant anti-botulinum scFv-pIII fusion protein (10^{12} cfu/ml; Amersdorfer *et al.*, 1997) as a negative control. The cell surface was stripped of phage antibodies using low-pH glycine buffer, the cells permeabilized and fixed, and intracellular phage detected with anti-M13 antibody. Remarkably, all cells showed strong intracellular staining when incubated with fd-F5 phage or with F5 phagemid but not when incubated with the anti-botulinum phagemid (Figure 1). This demonstrates the dependence of phage entry on the specificity of the scFv fused to pIII.

Preparation of ErbB2-binding phages and phagemids packaging a reporter gene for expression in eukaryotic cells

Two strategies were used to investigate whether F5 phage could deliver a reporter gene to mammalian cells leading to expression. To make monovalent phage containing a reporter gene, we cloned the gene for green fluorescent protein (GFP) driven by the CMV promoter into the phagemid vector pHEN-F5 generating the vector pHEN-F5-GFP (Figure 2, upper panel). *Escherichia coli* TG1 containing pHEN-F5-GFP (ampicillin resistant) were infected with helper phage (kanamycin resistant) and high titers of monovalent F5-GFP phagemids were obtained (5.0×10^{10} ampicillin-resistant cfu/ml of culture supernatant). The ratio of packaged phagemid DNA *versus* helper phage DNA (ampicillin *versus* kanamycin resistant cfu) was determined to be 100:1. To make multivalent phage containing a reporter gene, fd-F5-GFP phage were generated by infecting *E. coli* TG1 carrying the pcDNA3-GFP phagemid (ampicillin-resistant) with fd-F5 phage (tetracycline-resistant), thus using fd-F5 phage as a helper phage. The fd-F5-GFP phage

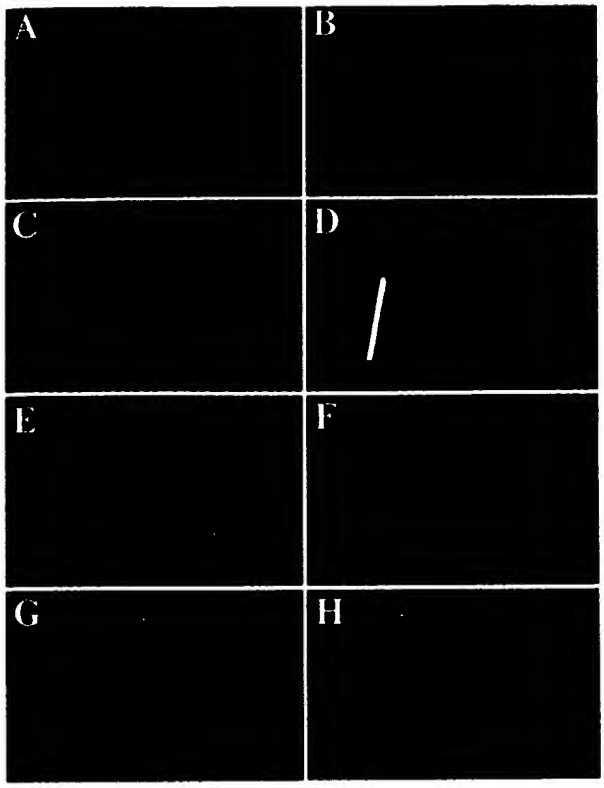

Figure 1. Internalization of anti-ErbB2 phagemids (monovalent) and phages (multivalent). SKBR3 cells (4.0×10^5) were incubated at 37°C with (a) and (b) no phage, (c) and (d) 10^{12} cfu/ml of anti-Botulinum phagemid (non specific phagemid), (e) and (f) 10^9 cfu/ml of anti-ErbB2 fd-F5-phage or (g) and (h) 10^{11} cfu/ml of anti-ErbB2 F5-phagemid. After 16 hours, the cell surface was stripped to remove the extracellular phages. Cells were fixed, permeabilized and stained with Hoechst dye (left column) to detect the cell nucleus and biotinylated anti-M13 antibody and streptavidin Texas-Red (right column) to detect intracellular phage particles.

titer was approximately 5.0×10^8 ampicillin resistant cfu/ml of culture supernatant. Lower phage titers result when fd is used as a helper phage because it lacks a plasmid origin of replication leading to interference from the phagemid f1 origin (Cleary & Ray, 1980). The ratio of packaged reporter gene DNA *versus* phage DNA (ampicillin *versus* tetracycline-resistant cfu) was 1:1. The lower ratio of reporter gene/helper genome when using fd as a helper phage is due to the presence of a fully functional packaging signal on the fd genome compared to the mutated packaging signal in VCS-M13 (Vieira & Messing, 1987). Both phage and phagemid preparations were assessed for SKBR3 cell binding (Figure 3). While both preparations bound SKBR3 cells, binding could be detected with as little as 10^8 cfu/ml of fd-F5-GFP phage cfu/ml (160 fM) compared to 10^{10} cfu/ml of F5-GFP phagemids (15 pM). Thus multivalent binding leads to an increase in the apparent binding constant of virus particles.

Targeted phagemid and phage-mediated g n transfer into ErbB2 xpressing breast cancer cells

To determine if ErbB2 binding phagemids were capable of targeted gene delivery, 2.0×10^5 SKBR3 cells (a breast tumor cell line expressing high levels of ErbB2) or 2.0×10^5 MCF7 cells (a low ErbB2 expressing breast tumor cell line) were incubated with 5.0×10^{11} cfu/ml F5-GFP phagemids at 37°C. SKBR3 cells express at least 27 to 170 times more ErbB2 than MCF7 cells (Lewis *et al.*, 1993). Cells were analyzed for GFP expression by FACS after 48 hours (Figure 4(a)). Of the SKBR3 cells, 1.37% expressed GFP after incubation with F5-GFP phagemids (Figure 4(a6)). GFP expression was identical regardless of the orientation of the f1 packaging signal (data not shown), indicating that transcription/translation was proceeding *via* synthesis of the complementary DNA strand. GFP expression was not detected in SKBR3 cells incubated with no phage or with helper phage packaging the reporter gene (Figure 4(a4) and 4(a5)). Expression was also not seen in MCF7 cells incubated with no phage, helper phage or pHEN-F5-GFP, indicating the requirement of ErbB2 expression for targeted gene delivery (Figure 4(a1), 4(a2) and 4(a3)). Since gene transfer applications are likely to involve targeting of specific cells in a heterogeneous cell population, we performed the same experiment on a co-culture of SKBR3 and MCF7 cells (Figure 4(b)). Cells were stained for ErbB2 expression to discriminate MCF7 from SKBR3 cells and the GFP expression of each subpopulation was analyzed by FACS. Only SKBR3 cells (1.91%) expressed GFP. Similar results were found using F5-GFP phages instead of F5-GFP phagemids (data not shown). These data confirm that fd-F5-GFP phage and F5-GFP phagemid-mediated gene delivery is restricted to ErbB2-over-expressing cells and can be targeted to such cells in the presence of non-expressing cells.

Characterization of phage-mediated gene transfer

To determine the dose-response characteristics of phage-mediated gene transfer, SKBR3 cells were incubated for 60 hours with increasing amounts of fd-F5-GFP phage or F5-GFP phagemids and the percentage of GFP-positive cells determined (Figure 5(a) and 5(b)). The minimal phage concentration required for detection of a significant number of GFP positive cells (Figure 5(a)) was approximately 4.0×10^7 cfu/ml for fd-F5-GFP phage (0.13%) and 1.0×10^{10} cfu/ml for F5-GFP phagemid (0.12%). The values correlate closely with the binding curves (Figure 3) and indicate that multivalent phage are 100 to 1000 time more efficient than phagemids in terms of gene expression. No significant number of positive cells were observed with up to 4.0×10^{13} cfu/ml of helper phage packaging the reporter gene. For both phage and phagemid, the percentage of GFP-

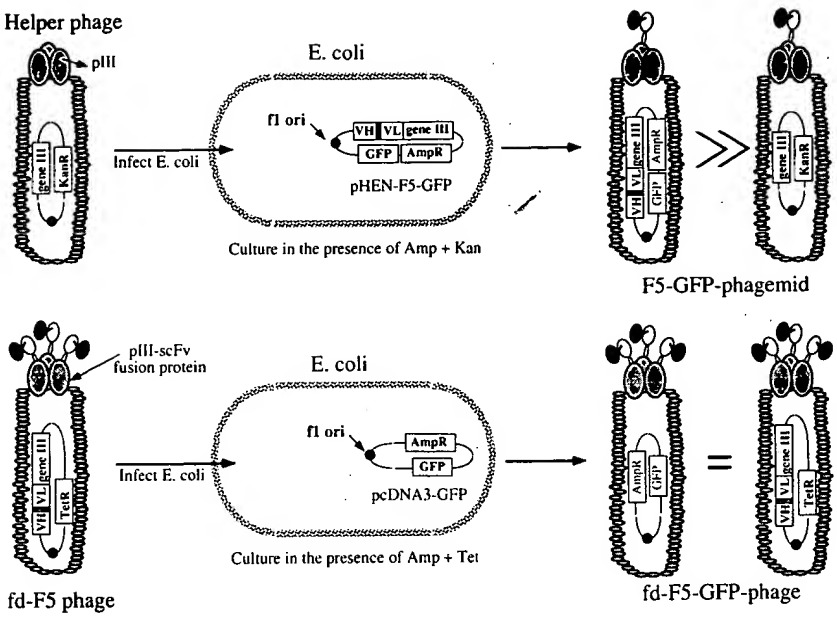

Figure 2. Strategies for producing anti-ErbB2 phagemids and phages packaging an eukaryotic reporter gene. Upper panel: helper phage are used to infect TG1 containing pHEN-F5-GFP, a phagemid composed of an f1 origin of replication (f1 ori), the anti-ErbB2 F5 scFv gene fused to gene III and a eukaryotic GFP reporter gene driven by the CMV promoter. Phages recovered from the culture supernatant display an average of 1 scFv-pIII fusion protein and 99% of them package the GFP reporter gene. Lower panel: the anti-ErbB2 F5 scFv gene is cloned into the fd phage genome for expression as a scFv-pIII fusion. fd-F5 phages are used to infect TG1 containing a GFP reporter phagemid vector (pcDNA3-GFP). Phages purified from the culture supernatant display multiple scFv-pIII fusion protein and approximately 50% package the GFP reporter gene.

positive cells increased with phage concentration with no evidence of a plateau. The maximum values achieved were 2% of cells for fd-F5-GFP phage and 4% for F5-GFP phagemids and appear to be limited by the phage titer applied (1.5×10^9 cfu/ml and 4.0×10^{12} cfu/ml, respectively). The amount of GFP expressed per cell (estimated by the mean fluorescent intensity (MFI), Figure 5(b)) also increased with phage concentration, with a small number of cells showing expression with phage titers as low as 2.0×10^7 cfu/ml (fd-F5-GFP phage) to 1.0×10^{10} cfu/ml (F5-GFP phagemid).

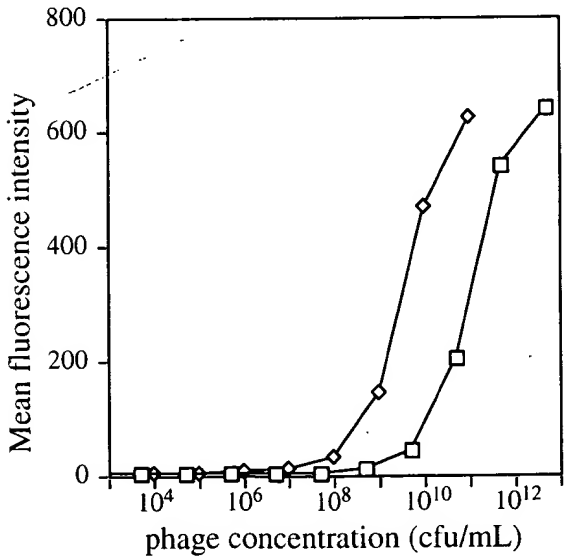

Figure 3. Comparison of anti-ErbB2 phagemid and phage binding on cells: 10^5 ErbB2 expressing SKBR3 cells were incubated with increasing concentrations of F5-phagemids (□) or fd-F5-phage (◇) at 4°C for one hour. Cell surface-bound phages were detected with biotinylated anti-M13 and streptavidin-PE. Binding was detected by FACS and the results are expressed as mean fluorescent intensity (MFI).

To compare the yield of gene expression obtained with phage to traditional transfection methods, single-stranded (ssDNA) or double-stranded DNA (dsDNA) was transfected into SKBR3 using lipofectamine. Per microgram of ssDNA, efficiency of phagemid-mediated gene delivery (approximately 1%) was comparable to lipofectamine transfection of ssDNA (0.98%) and dsDNA (1.27%; Table 1). Efficiency was approximately 500-fold higher for phage-mediated transfection, with 2.25 ng of ssDNA resulting in transfection of 0.87% of cells.

To determine the time-course of gene expression, 5.0×10^{11} cfu/ml of F5-GFP phagemid was incubated with SKBR3 cells. After 48 hours, the culture medium was replaced by fresh medium. GFP-expressing cells can be detected within 24 hours after phage are applied and the percentage of positive cells increases linearly with increasing time to a maximum of 4.5% by 120 hours (Figure 5(c)). The GFP content of the positive cells, as estimated by the MFI, increases up to 96 hours (Figure 5(d)). After 96 hours, the number of GFP-positive cells continues to increase but the MFI decreases, probably due to the repartition of GFP molecules to daughter cells during cell division.

Discussion

We demonstrate that filamentous phage displaying an anti-ErbB2 scFv antibody fragment as a genetic fusion with the minor coat protein pIII can be directly targeted to mammalian cells expressing

the specificity of the scFv. Such phage undergo receptor-mediated endocytosis and enter an intracellular trafficking pathway that ultimately leads to reporter gene expression. This is a remarkable finding, demonstrating that prokaryotic viruses can be re-engineered to infect eukaryotic cells, resulting in expression of a reporter gene inserted into the bacteriophage genome. Gene expression was detected with as few as 2.0×10^7 cfu of phage and increased with increasing phage titer up to 4% of cells. Multivalent display decreased the threshold for detectable gene expression approximately 500-fold compared to monovalent display, most likely due to an increase in the functional affinity and an increased rate of receptor-mediated endocytosis from receptor crosslinking. The maximum percentage of cells transfected, however, was higher for monovalent display (phagemid) due to the significantly higher phage titer generated. The lower titer of multivalent phage is due to interference of the f1 origin of replication on the reporter phagemid with the fd phage antibody origin of replication (Cleary & Ray, 1980).

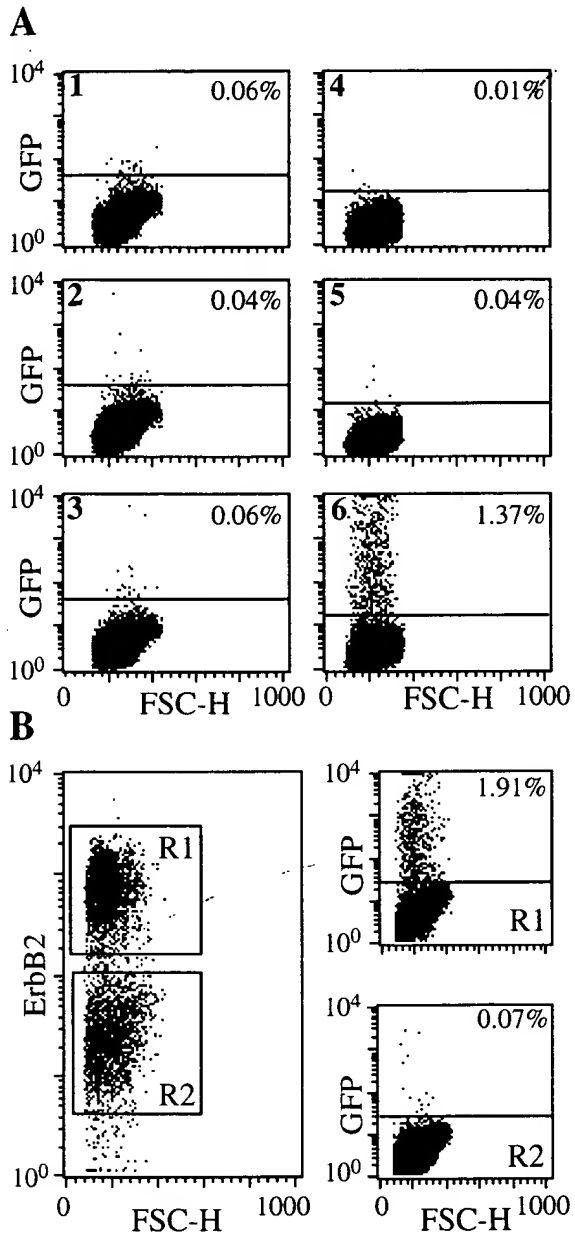

Figure 4. Phagemid-mediated gene transfer in breast cancer cell lines. (a) (1-3) 2.0×10^5 MCF7 (low ErbB2 expression) or (4-6) 2.0×10^5 SKBR3 (high ErbB2 expression) cells grown in six-well plates were incubated with (1 and 4) no phage, (2 and 5) 5.0×10^{12} cfu/ml of helper phage packaging GFP, or (3 and 6) 5.0×10^{11} cfu/ml of F5-GFP-phagemids for 48 hours. Cells were trypsinized and GFP detected by FACS. (b) An equal number of MCF7 and SKBR3 cells (1.0×10^5) were grown together and incubated with 5.0×10^{11} cfu/ml of F5-GFP-phagemids for 48 hours. Cells were trypsinized and stained for ErbB2 expression using 4D5 antibody and rhodamine-conjugated sheep anti-mouse Ig to discriminate SKBR3 (region R1) and MCF7 (region R2) cells. The GFP content of each subpopulation was determined by FACS.

Targeted infection of mammalian cells using phage that bind endocytosable receptors is likely to be a general phenomenon. For example, fusing an anti-transferrin receptor scFv to gene III of pHEN-GFP results in GFP expression in 10% of MCF7 cells, 4% of SKBR3 cells, 1% of LNCaP cells and 1% of primary melanoma cells (M. Huie & J.D.M., unpublished results). Similarly, targeted GFP gene delivery to FGF receptor-expressing cells using biotinylated phage and a streptavidin-FGF fusion molecule was recently reported (Larocca *et al.*, 1998). However, direct genetic fusion of the targeting molecule *via* gene III may be more efficient than using adapter molecules. Thus, while the maximum percentage of cells transfected using the FGF-adapter molecule was not reported, we estimate it to be only 0.03% of FGF expressing L6 rat myoblasts based on the number of cells infected, the time after infection to the measurement of gene expression and the number of cells expressing GFP. While a greater frequency of expression (0.5%) was seen in COS-1 cells, this results from the presence of large T antigen and SV40-mediated DNA replication and thus is not generalizable to most cells.

The approach we describe represents a novel method to discover ligands for targeted intracellular drug or gene delivery. Phage antibody or peptide libraries are first selected for endocytosis by mammalian cells (Barry *et al.*, 1996; Becerril *et al.*, 1999, M.-A.P. *et al.*, unpublished results) or for binding to purified antigen, cells, tissues or organs. After subcloning the selected scFv genes into the pHEN-GFP vector, phage produced from individual colonies can be directly screened for gene expression. This is possible, since expression can be detected with as little as 1.0×10^{10} cfu of phagemids. This permits direct identification of endocytosed scFv and the subset of receptor antibodies that undergo proper trafficking for gene expression. If multivalent display is necessary for efficient endocytosis, the scFv genes can be subcloned into fd-Sfi-Not, which is then used to rescue

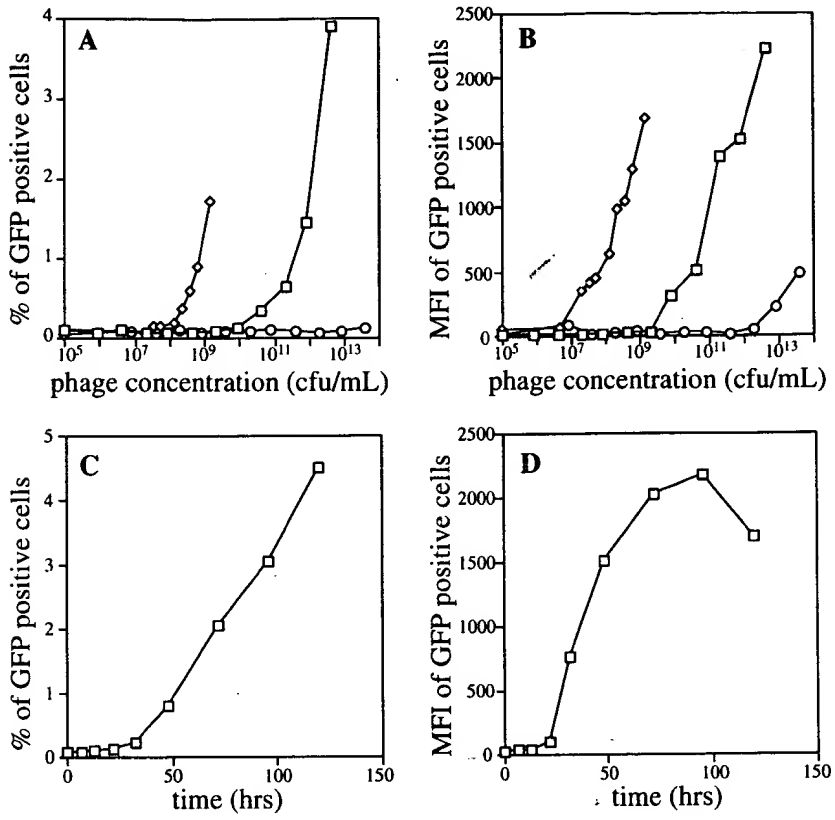

Figure 5. (a) and (b) Concentration-dependence of phagemid and phage-mediated GFP expression in SKBR3 cells. 5.0×10^4 cells were grown in 24-well plates and incubated with increasing concentrations of F5-GFP-phagemids (□), fd-F5-GFP-phage (◇) or GFP-helper phage (○). After 60 hours, the cells were trypsinized and analyzed by FACS for GFP expression. (c) and (d) Time-dependence of phagemid-mediated GFP expression in SKBR3 cells. The 5.0×10^4 cells were incubated with 5×10^{11} cfu/mL of F5-GFP-phagemid and analyzed for GFP expression by FACS. For incubation times longer than 48 hours, the phage were added to 2.5×10^4 cells and the culture medium was replaced by fresh medium after 48 hours of incubation. The results are expressed as (a) and (c) percentage of GFP-positive cells, and (b) and (d) MFI of the GFP-positive cells.

the reporter phagemid. Use of scFv-fd phage also allows the targeting of a large number of different reporter genes in various expression vectors, since many commercially available mammalian vectors contain f1 origins of replication. As such, antibody-targeted phage might prove to be useful transfection reagents, especially for cells that are difficult to transfect by standard techniques.

It may also prove possible to use this approach to directly select, rather than screen, antibodies for targeted gene delivery. For example, mammalian cells are incubated with a phage antibody library containing the GFP gene, and then sorted based on GFP expression using FACS. Phage antibody DNA would be recovered from the mammalian cytoplasm by cell lysis and used to transfect *E. coli* and prepare more phage for another round of selection. If the quantities of recoverable phage DNA are inadequate, inclusion of the neomycin gene in the pHEN-GFP vector would permit selection of GFP-expressing mammalian cells using G418 (Larocca *et al.*, 1998).

Finally, this system has promise as a targetable *in vitro* or *in vivo* gene therapy vehicle. The main limitations are infection efficiency, pharmacokinetics and immunogenicity. With respect to infection efficiency, values achieved by targeted phage in this study (8.0×10^4/ml of phage preparation) are not dissimilar to values reported for targeted retrovirus ($10^3 - 10^5$/ml of virus; Kasahara *et al.*,

Table 1. Transfection efficiencies in SKBR3 cells

Transfection method	Reporter plasmid		Amount of reporter plasmid DNA	% of GFP positive cells
F5-phagemid-mediated	pHEN-F5-GFP		15 μg	3.84
			3.1 μg	1.44
			0.78 μg	0.64
fd-F5-phage- mediated	pcDNA3-GFP		5 ng	1.69
			2.25 ng	0.87
			1.25 ng	0.57
Helper phage- mediated	pN_2GFP		100 μg	0.12
			20 μg	0.07
			5 μg	0.06
Lipofectamine	pN_2GFP	dsDNA	1 μg	1.27
		ssDNA	1 μg	0.98

[a] Cells were analysed 48 hours after transfection for GFP expression using FACS. Results are expressed in % of GFP positive cells. For phage, the amount of reporter plasmid was calculated from the plasmid size and the number of ampicillin (pHEN-F5-GFP or pcDNA3-GFP) or kanamycin (pN_2GFP) resistant colonies. Mock transfected cells contained an average of 0.05 % GFP-positive cells.

1994; Somia *et al.*, 1995) but less than reported for adenovirus targeting strategies (up to 100% of cells; Douglas *et al.*, 1996; Watkins *et al.*, 1997). The factors limiting higher infection efficiencies, however, are likely to differ between the systems. Thus while the percentage of cells infected by retrovirus is significantly higher than observed for bacteriophage, infection is limited by the problems encountered in producing large numbers of virus that can enter the cell. Since all cells take up the targeted phage (Figure 1), gene expression is limited by one or several post-uptake events (e.g. degradation of phage to release DNA, endosomal escape, nuclear targeting or transcription). More detailed study of the fate of the phage and its DNA is likely to suggest where the block lies, permitting engineering of phage to increase infection efficiency. For example, endosomal escape could be enhanced by co-administering replication-defective adenovirus (Curiel *et al.*, 1991) or incorporating endosomal escape peptides (Wagner *et al.*, 1992) or proteins (Fominaya & Wels, 1996) into the phage major coat protein pVIII. Alternatively, infection efficiency could be increased combinatorially by creating scFv-targeted libraries of pVIII mutants and selecting for increased gene expression. With respect to pharmacokinetics, though not extensively studied, concentrations of phage are much higher in the intravascular space than in tissue (Rajotte *et al.*, 1998). This would not affect *in vitro* phage gene therapy, but might limit *in vivo* uses to those targeting the vasculature. This still leaves numerous applications, including those where neovascularization plays a role, such as cancer. With respect to immunogenicity, it is likely that phage will be immunogenic, thus limiting the number of times that phage could be administered *in vivo*. Alternatively, it might prove possible to evolve the major coat protein pVIII to reduce or eliminate immunogenicity, for example by negatively selecting a pVIII library on immune serum (Jenne *et al.*, 1998).

Experimental protocol

Anti-ErbB2 F5 scFv

An anti-ErbB2 scFv (F5) in the vector pHEN-1 (Hoogenboom *et al.*, 1991; pHEN-F5) was obtained by selecting a non-immune phage antibody library (Sheets *et al.*, 1998) on ErbB2-expressing SKBR3 cells followed by screening for binding on recombinant ErbB2 extracellular domain (ECD) (M.-A.P. *et al.*, unpublished results). The native F5 scFv binds ErbB2 ECD with $K_d = 1.6 \times 10^{-7}$ M as determined by surface plasmon resonance in a BIAcore (Schier *et al.*, 1996).

Phage and phag mid vectors

pcDNA3-GFP (6.1 kbp) was obtained by subcloning the *Hin*dIII/*Not*I fragment of pN_2EGFP (4.7 kbp; Clontech) into the pcDNA3-HisB/LacZ (Invitrogen) *Hin*dIII/*Not*I backbone. An fd-F5-phage vector was constructed by subcloning the *Sfi* I/*Not* I scFv-F5 insert from pHEN-1 into the *Sfi* I/*Not*I sites of fd-Sfi/Not (constructed from fd-tet-DOG (Clackson *et al.*, 1991) by changing the *ApaL*1 cloning site in the gene III leader to *Sfi*I; a gift from Dr Andrew Griffiths, MRC, Cambridge). The pHEN-F5-GFP phagemid vector (6.8 kbp) was obtained by subcloning the 1.6 kbp pN_2EGFP-blunted *Ase*I/*Afl*II fragment into the blunted *Eco*RI site of pHEN-F5. The orientation of the insert was analyzed by *Not*I restriction digest.

Cell line culture and transfection

SKBR3 and MCF7 were grown in RPMI complemented with 10% fetal bovine serum (FBS) (Hyclone). The 50% confluent SKBR3 cells grown in six-well plates were transfected with 1 μg of DNA per well using lipofectamine (GIBCO BRL) as recommended by the manufacturer. pN_2EGFP dsDNA was prepared by alkaline lysis using the Maxiprep Qiagen Kit (Qiagen Inc.). ssDNA was extracted from 500 μl of phagemid preparation (see below) by two extractions with phenol followed by precipitation in ethanol. DNA was quantified by spectophotometry with 1.0 A_{260} equal to 40 μg/ml for ssDNA or 50 μg/ml for dsDNA. For GFP detection, cells were detached using a trypsin-EDTA mix (GIBCO BRL) and analyzed on a FACScan (Becton Dickinson).

Phagemid and phage preparation

pHEN-F5, pHEN-F5-GFP, pcDNA3-GFP or pN_2EGFP phagemids were prepared from *E. coli* TG1 by superinfection with VCS-M13 helper phage (Stratagene) as described (Marks *et al.*, 1991). Fd-F5-phage were prepared from *E. coli* TG1 as described (McCafferty *et al.*, 1990). F5-GFP-phage and F5-LacZ-phage were prepared by superinfection of *E coli* TG1 containing pcDNA3-GFP with fd-F5-phage. Virus particles were purified from the culture supernatant by two precipitations in polyethylene glycol (Sambrook *et al.*, 1990) resuspended in phosphate-buffered saline, pH 7.4 (PBS), filtered through a 0.45 μm filter and stored at 4 °C. Alternatively, the preparations were submitted to an additional CsCl ultracentrifugation step (Smith & Scott, 1993). The ratio of packaged helper phage DNA *versus* phagemid DNA was determined by titering (Sambrook *et al.*, 1990) the phage for ampicillin and kanamycin-resistance (for helper phage-rescued pHEN-F5) or ampicillin and tetracycline-resistance (for fd-F5 phage-rescued pcDNA3-GFP).

Phage FACS

Cells were trypsinized, washed with FACS buffer (PBS containing 1% FBS) and resuspended at 10^6 cells/ml in the same buffer. The staining procedure was performed on ice with reagents diluted in FACS buffer. Aliquots (100 μl) of cells were distributed in conical 96-well plates (Nunc), centrifuged at 300 *g* and the cell pellets resuspended in 100 μl of serial dilutions of phage or phagemid preparation and incubated for one hour. Cells were centrifuged and washed twice, the cell pellets resuspended in 100 μl of biotinylated anti-M13 antibody (5 Prime, 3 Prime Inc.: diluted 1/400) and incubated for 45 minutes. Cells were washed as above, resuspended in 100 μl of streptavidin-Phycoerythrin (Jackson Inc.; diluted 1/400) and incubated for 20 minutes. After a final wash, the cells were analyzed by FACS.

Immunofluorescence

Cells were grown on coverslips to 50% confluency in six-well plates. Phage preparation (less than 10% of the culture medium) was added and the cells were incubated for 16 hours. The coverslips were washed six times with PBS, three times for ten minutes with glycine buffer (50 mM glycine (pH 2.8), 500 mM NaCl), neutralized with PBS and fixed with PBS-4% paraformaldehyde for five minutes at room temperature. Cells were permeabilized with cold acetone for 30 seconds, saturated with PBS-1% BSA and incubated with anti-M13 antibody (diluted 1/300 in the saturation solution) followed by streptavidin-Texas Red (Amersham; diluted 1/300 in the saturation solution). Coverslips were analyzed with a Zeiss Axioskop fluorescent microscope (Zeiss).

Bacteriophage mediated cell infection

CsCl phage preparations were diluted at least tenfold in cell culture medium, filtered through a 0.45 μm filter and added to 30% to 80% confluent cells. After incubation, the cells were trypsinized, washed with FACS buffer and analyzed for GFP expression by FACS. In the experiments where MCF7 and SKBR3 were co-cultured, ErbB2 expression was quantified by FACS using the anti-ErbB2 mouse mAb 4D5 which binds ErbB2 ECD (a gift from Paul Carter, Genentech, Inc.; 10 μg/ml for one hour), biotinylated sheep anti-mouse immunoglobulins (Amersham) and streptavidin-Phycoerythrin.

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